Differential Modulation by Copper and Zinc of $P2X_2$ and $P2X_4$ Receptor Function

KEMING XIONG, ROBERT W. PEOPLES, JENNIFER P. MONTGOMERY, YISHENG CHIANG, RANDALL R. STEWART, FORREST F. WEIGHT, AND CHAOYING LI

Laboratory of Molecular and Cellular Neurobiology, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland 20892–8115

Keming Xiong, Robert W. Peoples, Jennifer P. Montgomery, Yisheng Chiang, Randall R. Stewart, Forrest F. Weight, and Chaoying Li. Differential Modulation by Copper and Zinc of $P2X_2$ and $P2X_4$ Receptor Function. *J. Neurophysiol.* 81: 2088–2094, 1999. The modulation by Cu^{2+} and Zn^{2+} of $P2X_2$ and $P2X_4$ receptors expressed in *Xenopus* oocytes was studied with the two-electrode, voltage-clamp technique. In oocytes expressing $P2X_2$ receptors, both Cu^{2+} and Zn^{2+}, in the concentration range 1–130 μM, reversibly potentiated current activated by submaximal concentrations of ATP. The Cu^{2+} and Zn^{2+} concentrations that produced 50% of maximal potentiation (EC_{50}) of current activated by 50 μM ATP were 16.3 ± 0.9 (SE) μM and 19.6 ± 1.5 μM, respectively. Cu^{2+} and Zn^{2+} potentiation of ATP-activated current was independent of membrane potential between −80 and +20 mV and did not involve a shift in the reversal potential of the current. Like Zn^{2+}, Cu^{2+} increased the apparent affinity of the receptor for ATP, as evidenced by a parallel shift of the ATP concentration-response curve to the left. However, Cu^{2+} did not enhance ATP-activated current in the presence of a maximally effective concentration of Zn^{2+}, suggesting a common site or mechanism of action of Cu^{2+} and Zn^{2+} on $P2X_2$ receptors. For the $P2X_4$ receptor, Zn^{2+}, from 0.5 to 20 μM enhanced current activated by 5 μM ATP with an EC_{50} value of 2.4 ± 0.2 μM. Zn^{2+} shifted the ATP concentration-response curve to the left in a parallel manner, and potentiation by Zn^{2+} was voltage independent. By contrast, Cu^{2+} in a similar concentration range did not affect ATP-activated current in oocytes expressing $P2X_4$ receptors, and Cu^{2+} did not alter the potentiation of ATP-activated current produced by Zn^{2+}. The results suggest that Cu^{2+} and Zn^{2+} differentially modulate the function of $P2X_2$ and $P2X_4$ receptors, perhaps because of differences in a shared site of action on both subunits or the absence of a site for Cu^{2+} action on the $P2X_4$ receptor.

INTRODUCTION

The P2X receptors are ligand-gated membrane ion channels that are activated by extracellular ATP. These receptor channels received recent attention because of their potential importance in the central and peripheral nervous systems. Activation of P2X receptors elicits excitatory postsynaptic currents or excitatory postsynaptic potentials in both central and peripheral neurons (Bardoni et al. 1997; Edwards et al. 1992, 1997; Evans et al. 1992; Galligan and Bertrand 1994; Gu and MacDermott 1997; Pankratov et al. 1998; Silinsky et al. 1992) and excitatory junction potentials in smooth muscle cells (Sneddon et al. 1982). Activation of P2X receptors also mediates excitatory responses in a variety of central and peripheral neurons (Bean 1990; Fieber and Adams 1991; Khakh et al. 1995; Krishtal et al. 1983; Li et al. 1993, 1997a; Shen and North 1993; Ueno et al. 1992). P2X receptors were found to be widely distributed in the CNS, including cerebral cortex, hippocampus, thalamus, hypothalamus, midbrain, cerebellum, and spinal cord, and in sensory and sympathetic ganglia in the peripheral nervous system (Collo et al. 1996).

Like other neurotransmitter-gated membrane ion channels, P2X receptors in neurons are sensitive to a number of endogenous agents, including Zn^{2+} (Cloues et al. 1993; Li et al. 1993, 1997a), Cu^{2+} (Li et al. 1996a), H^+ (Li et al. 1996b), Mg^{2+}, and Ca^{2+} (Krishtal and Marchenko 1984; Li et al. 1997b; Nakazawa and Hess 1993) as well as other neurotransmitters or neuromodulators, such as substance P (Hu and Li 1996; Wildman et al. 1997). Recent studies revealed that these substances can produce differential effects on P2X receptors in neurons. For instance, in rat nodose ganglion neurons, low micromolar concentrations of Zn^{2+} and Cu^{2+} enhance ATP-activated current in the majority of neurons but have no effect in a subset of neurons (Li et al. 1993, 1996a). On the other hand, in bullfrog dorsal root ganglion neurons, low micromolar concentrations of Zn^{2+} inhibit ATP-activated current (Li et al. 1997a). Extracellular protons markedly potentiate ATP-activated current in the majority of neurons from rat nodose ganglion but do not alter ATP-activated current in a subset of these neurons (Li et al. 1996a,b). Similarly, Mg^{2+} inhibits ATP-activated current in most but not all neurons from rat nodose ganglion (Li et al. 1997b). The molecular mechanisms underlying the diverse effects of these modulators, however, remain to be determined.

At least seven P2X receptor subunits, designated $P2X_1$–$P2X_7$, were cloned to date (Buell et al. 1996a). Each of these subunits can form ATP-selective homomeric cation channels when expressed in *Xenopus* oocytes or cell lines. Characterization of the properties of recombinant P2X receptor subunits should prove to be a useful first step in resolving the disparate effects of modulators on P2X receptors in neurons. In this regard, results of recent studies revealed a differential modulation of P2X receptor subunits by endogenous agents. For instance, extracellular Ca^{2+} strongly inhibits the $P2X_2$ subunit but not the $P2X_1$ subunit (Evans et al. 1996). In addition, low micromolar concentrations of Zn^{2+} potentiate $P2X_2$ and $P2X_4$ subunits (Brake et al. 1994; Garcia-Guzman et al. 1997; Séguéla et al. 1996; Wildman et al. 1998) but inhibit the $P2X_7$ subunit (Virginio et al. 1997). Morever, extracellular protons

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inhibit $P2X_1$, $P2X_3$, $P2X_4$, and $P2X_7$ subunits but potentiate the $P2X_2$ subunit as well as the $P2X_2$ and $P2X_3$ heteromeric receptor (Stoop et al. 1997). To characterize further the physiological regulation of P2X receptor subunits, we investigated the effects of Cu^{2+} and Zn^{2+} and their possible interactions on recombinant $P2X_2$ and $P2X_4$ receptors.

METHODS

Preparation of cRNA and expression of receptors

cRNA was synthesized in vitro from a linearized cDNA template with T7 RNA polymerase in the presence of the cap analogue 7 mGpppG and was injected into *Xenopus* oocytes with a pressurized microinjection device (PV 800 Pneumatic Picopump, World Precision Instruments; Sarasota, FL). Mature *X. laevis* frogs were anesthetized by immersion in water containing 3-aminobenzoic acid ethyl ester (2 g/l). Oocytes were excised, mechanically isolated into clusters of four to five oocytes, and shaken in a water bath in two changes of 0.2% collagenase A in a solution containing (in mM) 83 NaCl, 2 KCl, 1 $MgCl_2$, and 5 HEPES, pH 7.4, for 1 h each. Each oocyte was injected with a total of 10 ng of RNA in 50 nl of diethylpyrocarbonate-treated water and was incubated at 17°C for 2–5 days in modified Barth's saline containing sodium pyruvate (2 mM), penicillin (10,000 U/l), streptomycin (10 mg/l), gentamycin (50 mg/l), and theophylline (0.5 mM).

The care and use of animals in this study was approved by the Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism (protocol no. LMCN-SP-05) in accordance with National Institutes of Health guidelines.

Electrophysiological recording

Two-electrode, voltage-clamp recording was performed at room temperature with a Geneclamp (Axon Instruments; Foster City, CA) amplifier. Oocytes were placed in a recording chamber and impaled with two sharp electrodes filled with 3 M KCl. Electrode tip resistances were in the range 0.5–1.5 MΩ. Oocytes were usually voltage clamped at −70 mV, except as indicated. Currents were recorded on a pen recorder (Model RS3400, Gould; Valley View, OH). Oocytes were constantly superfused at the rate of ~2.5 ml/min with bathing medium containing (in mM) 95 NaCl, 2 KCl, 2 $CaCl_2$, and 5 HEPES, pH 7.4. Solutions of ATP (as the sodium salt) and Cu^{2+} (as $CuCl_2$) or Zn^{2+} (as $ZnCl_2$) were prepared daily in extracellular medium. Solutions of ATP and Cu^{2+} or Zn^{2+} were administered via the bathing solution, which was applied by gravity flow from a 0.5-mm silica tube connected to a seven-barrel manifold. Solutions were changed via manually switched solenoid valves. At least 5 min was allowed to elapse between agonist applications.

Drugs and chemicals

All of the drugs and chemicals used in these experiments were purchased from Sigma Chemical (St. Louis, MO), except $CuCl_2$, which was purchased from Aldrich Chemical (Milwaukee, WI), and the salts, which were purchased from Mallinckrodt (Paris, KY).

Estimation of Zn^{2+} concentration

Concentrations of free Zn^{2+} were estimated with the program "Bound and Determined" (Brooks and Storey 1992), which compensates for variation in temperature, pH, and ionic strength. Values for Mn^{2+} were used as estimates of Zn^{2+} concentrations because ATP has similar affinities for Mn^{2+} and Zn^{2+} (16 vs. 14 μM) (Sillen and Martell 1964), and the software does not directly calculate Zn^{2+} concentration. All concentrations of ATP, Zn^{2+}, and Cu^{2+} given are total concentrations unless stated otherwise.

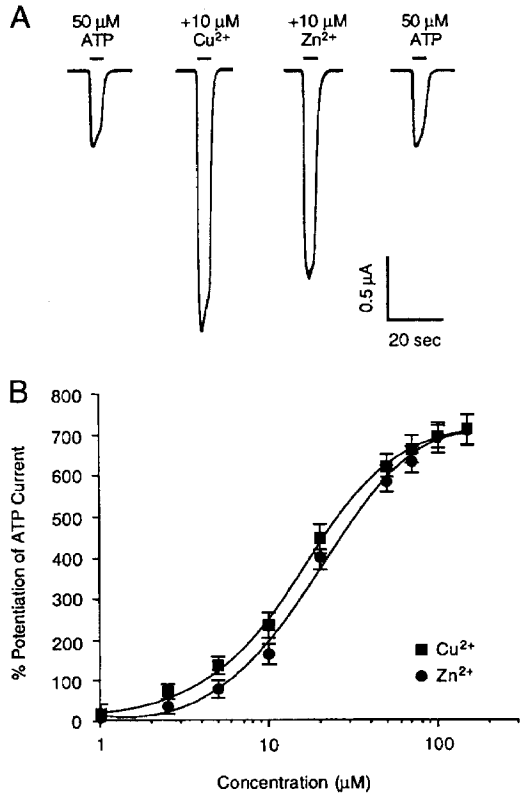

FIG. 1. Potentiation by Cu^{2+} and Zn^{2+} of ATP-activated current mediated by $P2X_2$ receptors. *A*: records of current activated by 50 μM ATP in the absence and the presence of 10 μM Cu^{2+} or Zn^{2+}. Records are sequential current traces (from left to right) obtained from a single oocyte. Solid bar above each record indicates time of agonist application in the absence or presence of Cu^{2+} or Zn^{2+}, as labeled. *B*: concentration-response curves for Cu^{2+} (■) and Zn^{2+} (●) potentiation of current activated by 50 μM ATP. Each point is the average of 6–14 cells; error bars not visible are smaller than the size of the symbols. The curves shown are the best fit of the data to the equation described in METHODS. Fitting the data to this equation yielded EC_{50} values of 16.3 ± 0.9 μM and 19.6 ± 1.5 μM, slope factors of 1.5 and 1.6, and E_{max} values of 845 ± 16% and 837 ± 26% of control for Cu^{2+} and Zn^{2+}, respectively. These values are not significantly different (ANOVA, $P > 0.1$).

Data analysis

Current amplitudes reported are peak values, and average values are expressed as means ± SE, with n equal to the number of cells studied. Data were statistically compared with Student's t-test or ANOVA as noted. Statistical analysis of concentration-response data was performed with the nonlinear curve-fitting program ALLFIT (DeLean at al. 1978), which uses an ANOVA procedure. Values reported for concentrations yielding 50% of maximal effect (EC_{50}) and slope factor (n) are those obtained by fitting the data to the equation

$$Y = E_{max}/[1 + (EC_{50}/X)^n]$$

where X and Y are concentration and response, respectively, and E_{max} is the maximal response.

RESULTS

Modulation of $P2X_2$ receptors by Cu^{2+} and Zn^{2+}

ATP, at concentrations of ≤500 μM, did not evoke detectable ion current in uninjected oocytes ($n = 6$, data not shown). Figure 1 illustrates the ATP-activated inward current in oocytes expressing $P2X_2$ receptors and the potentiation of that

current by extracellular Cu^{2+} and Zn^{2+}. As shown in Fig. 1*A*, the amplitude of inward current activated by 50 μM ATP was greatly enhanced by the application of 10 μM Cu^{2+}. To compare the effect of Cu^{2+} with that of Zn^{2+} (Brake et al. 1994; Wildman et al. 1998), potentiation of ATP-activated current by Zn^{2+} was also tested. At the same concentration, Zn^{2+} produced enhancement of ATP-activated current that was comparable with that of Cu^{2+} in the same oocyte. On average, in the same oocytes, 10 μM Cu^{2+} or 10 μM Zn^{2+} increased the amplitude of current activated by 50 μM ATP by 240 ± 32% (n = 12) or 167 ± 24% (n = 14), respectively. The enhancement by both divalent cations was concentration dependent between 1 and 130 μM (Fig. 1*B*). The EC_{50} values for Cu^{2+} and Zn^{2+} enhancement of current activated by 50 μM ATP were 16.3 ± 0.9 μM and 19.6 ± 1.5 μM, the slope factors were 1.5 and 1.6, and the maximal effects were 845 ± 16% and 837 ± 26% of control, respectively. The EC_{50}, slope factor, and E_{max} values obtained for Cu^{2+} did not differ significantly from those for Zn^{2+} (ANOVA, $P > 0.1$). Cu^{2+} or Zn^{2+} alone (1–130 μM) did not activate ion current in any oocytes tested (data not shown, n = 5).

Experiments performed to elucidate the mechanism by which Cu^{2+} augments ATP-activated current are shown in Fig. 2. As shown in Fig. 2*A*, the magnitude of Cu^{2+} potentiation decreased with increasing ATP concentration. On average, 5 μM Cu^{2+} increased the amplitude of the current activated by 10 and 100 μM ATP by 383 ± 28% (n = 6) and 15.3 ± 4% (n = 5), respectively. The graph in Fig. 2*B* shows the concentration-response curves for ATP-activated currents in the absence and presence of 5 μM Cu^{2+}. As can be seen, Cu^{2+} shifted the ATP concentration-response curve to the left, reducing the EC_{50} for ATP from 51.7 ± 1.9 μM in the absence of Cu^{2+} to 15.5 ± 0.3 μM in the presence of 5 μM Cu^{2+} (ANOVA, $P < 0.01$) without significantly changing the slope or maximal value (ANOVA, $P > 0.1$). The lack of effect of Cu^{2+} on the maximal value of the ATP concentration-response curve was apparently not because of chelation of Cu^{2+} by high concentrations of ATP, as increasing the Cu^{2+} concentration threefold, which would yield a calculated concentration of free Cu^{2+} greater than that required to produce potentiation (results for Zn^{2+} potentiation of $P2X_4$ receptors are described subsequently), did not potentiate current activated by 100 μM ATP (results not shown).

The influence of membrane potential on the potentiation by Cu^{2+} and Zn^{2+} of ATP-activated current was evaluated by constructing current-voltage relationships for ATP-activated current. Figure 3*A* shows the current-voltage relationship for current activated by 50 μM ATP in the absence and presence of 5 μM Cu^{2+}. Cu^{2+} produced a similar percentage enhancement of amplitude of current activated by ATP at membrane voltages between −80 and +20 mV and did not alter the reversal potential of ATP-activated current. In five of five cells tested, Cu^{2+} enhanced ATP-activated current in a voltage-independent manner (ANOVA, $P > 0.25$) and did not significantly change the reversal potential of ATP-activated current (Student's t-test, $P > 0.25$). Similarly, as shown in Fig. 3*B*, Zn^{2+} potentiation of ATP-activated current was voltage independent (ANOVA, $P > 0.25$, n = 4), and Zn^{2+} did not significantly change the reversal potential of ATP-activated current (Student's t-test, $P > 0.25$, n = 4).

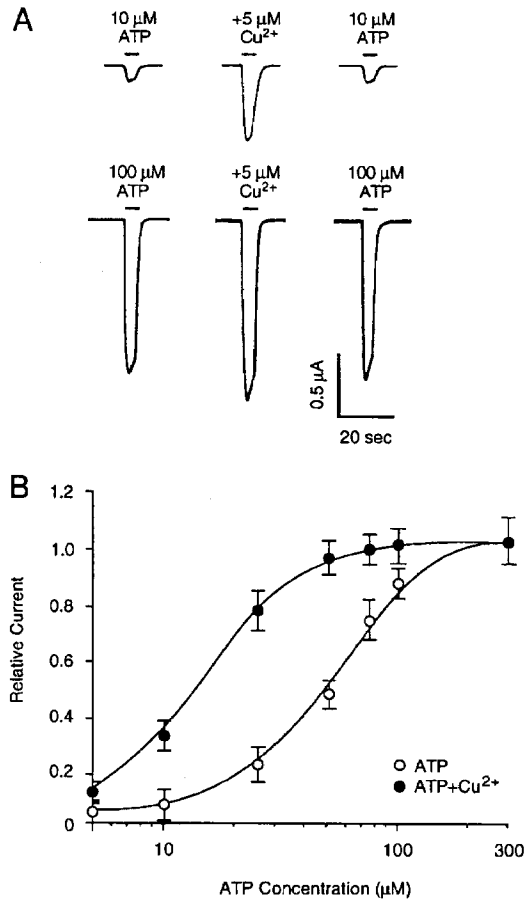

FIG. 2. Effect of ATP concentration on Cu^{2+} potentiation of ATP-activated current mediated by $P2X_2$ receptors. *A*: records showing currents activated by 10 μM ATP (*top traces*) and 100 μM ATP (*bottom traces*) before, during, and after application of 5 μM Cu^{2+} in a single oocyte. *B*: graph plotting the relative amplitude of ATP-activated current in the absence (○) and presence (●) of 5 μM Cu^{2+} as a function of ATP concentration. Amplitude is normalized to the current activated by 300 μM ATP in the absence of Cu^{2+}. Each data point is the average current from 5–8 cells. The curves shown are the best fits of the data to the equation described in METHODS. Cu^{2+} significantly decreased the EC_{50} for ATP from 51.7 ± 1.9 μM in the absence of Cu^{2+} to 15.5 ± 0.3 μM in the presence of 5 μM Cu^{2+} (ANOVA, $P < 0.01$).

Because Cu^{2+} and Zn^{2+} are closely related metals and have similar augmenting effects on ATP-activated current mediated by $P2X_2$ receptors, we hypothesized that they might act at a common binding site. Results of an experiment designed to test this hypothesis are shown in Fig. 4. A near-threshold concentration of ATP was used to obtain a current in the presence of a maximally effective concentration of Zn^{2+} that was lower in amplitude than the maximal ATP-activated current. In the cell shown in Fig. 4*A*, a maximally effective concentration of Zn^{2+} (130 μM) potentiated current activated by 4 μM ATP by 2,867%, and 10 μM Cu^{2+} increased the ATP-activated current by 1,467%. However, concomitant application of 130 μM Zn^{2+} and 10 μM Cu^{2+} failed to produce enhancement of ATP-activated current greater than that produced by Zn^{2+} alone. On average, the potentiation of ATP-activated current produced by Cu^{2+} and Zn^{2+} applied together was not different from that produced by Zn^{2+} applied alone (Student's t-test, $P > 0.25$, n = 5; Fig. 4*B*).

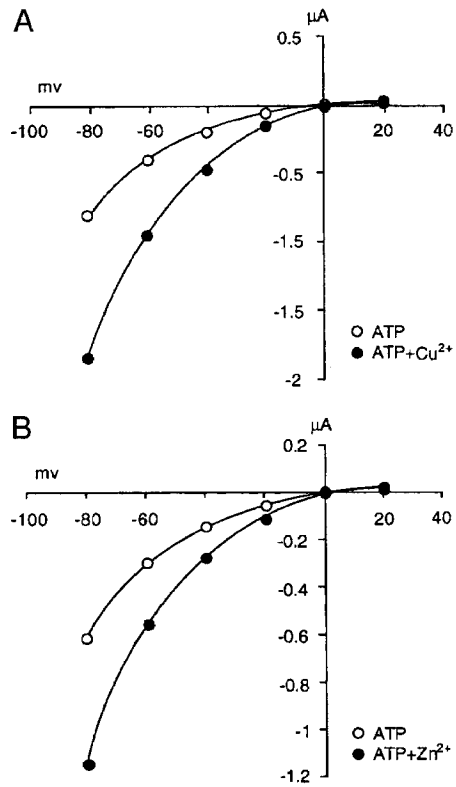

FIG. 3. Effect of membrane potential on potentiation by Cu^{2+} and Zn^{2+} of ATP-activated current mediated by $P2X_2$ receptors. *A*: current-voltage relationship for currents activated by 50 μM ATP in the absence (○) and the presence (●) of 5 μM Cu^{2+} at membrane potentials between −80 and +20 mV in a single cell. The average reversal potential of current activated by 50 μM ATP was −1 ± 4 mV in the absence and 1 ± 6 mV in the presence of 5 μM Cu^{2+}; these values are not significantly different (Student's *t*-test, $P > 0.25$, $n = 5$). In addition, the percentage potentiation of ATP-activated current by Cu^{2+} was not significantly different at holding potentials from −80 to +20 mV (ANOVA, $P > 0.25$, $n = 5$). *B*: current-voltage relationship for currents activated by 50 μM ATP in the absence (○) and the presence (●) of 5 μM Zn^{2+} at membrane potentials between −80 and +20 mV in a single oocyte. The average reversal potential of current activated by 50 μM ATP was −0 ± 5 mV in the absence and 0 ± 4 mV in the presence of 5 μM Zn^{2+}; these values are not significantly different (Student's *t*-test, $P > 0.25$, $n = 4$). In addition, the percentage potentiation of ATP-activated current by Zn^{2+} was not significantly different at holding potentials from −80 to +20 mV (ANOVA, $P > 0.25$, $n = 4$). Data in *A* and *B* are from different cells. Membrane potential was held at each value for 1 min before application of ATP in both *A* and *B*.

Modulation of $P2X_4$ receptors by Zn^{2+} and Cu^{2+}

The ATP-activated inward current in oocytes expressing $P2X_4$ receptors and the modulation of that current by extracellular Zn^{2+} and Cu^{2+} are illustrated in Fig. 5. As shown in Fig. 5*A*, 10 μM Zn^{2+} markedly increased the amplitude of current activated by 5 μM ATP. By contrast, the same concentration of Cu^{2+} did not affect current activated by the same concentration of ATP. Zn^{2+} potentiation of ATP-activated current was concentration dependent between 0.5 and 20 μM. The EC_{50} value for Zn^{2+} potentiation of current activated by 5 μM ATP was 2.4 ± 0.2 μM, the slope factor was 1.8, and the maximal effect was 214 ± 12% of control (Fig. 5*B*). Zn^{2+} alone (0.5–20 μM) did not activate ion current in any oocytes tested (data not shown, $n = 5$). In contrast to the potentiation of ATP-activated current by Zn^{2+}, Cu^{2+}, in the same concentration range, did not significantly affect ATP-activated current (ANOVA, $P > 0.25$; Fig. 5*B*). In addition, Cu^{2+} at a concentration of 50 μM did not potentiate ATP-activated current in oocytes expressing $P2X_4$ receptors (Student's *t*-test, $P > 0.5$, $n = 7$).

As the maximal potentiation by Cu^{2+} of ATP-activated current in $P2X_2$ receptors occurred at the lowest ATP concentration, we tested whether Cu^{2+} would potentiate the current activated by a near-threshold concentration of ATP in $P2X_4$ receptors. Results from one such experiment are illustrated in Fig. 6. In this experiment, 5 and 20 μM Cu^{2+} did not appreciably affect the current activated by 1.5 μM ATP. By contrast, 5 μM Zn^{2+} markedly enhanced ATP-activated current in the same cell. Similar results were obtained in five other experiments.

Figure 7*A* shows that Zn^{2+} shifted the ATP concentration-response curve to the left, reducing the EC_{50} value for ATP-activated current from 6.7 ± 1.3 μM in the absence of Zn^{2+} to 2.8 ± 0.2 μM in the presence of 5 μM Zn^{2+} (ANOVA, $P < 0.01$) without changing the slope or maximal value (ANOVA, $P > 0.1$). The lack of effect of Zn^{2+} on the maximal value of the ATP concentration-response curve did not appear to be due to chelation of Zn^{2+} by high concentrations of ATP, as addition of 10 μM Zn^{2+} yielded a calculated free Zn^{2+} concentration of 6.9 μM, but did not potentiate current activated by 100 μM ATP (results not shown). This calculated concentration of free Zn^{2+} is substantially greater than that produced by 5 μM Zn^{2+} in the presence of

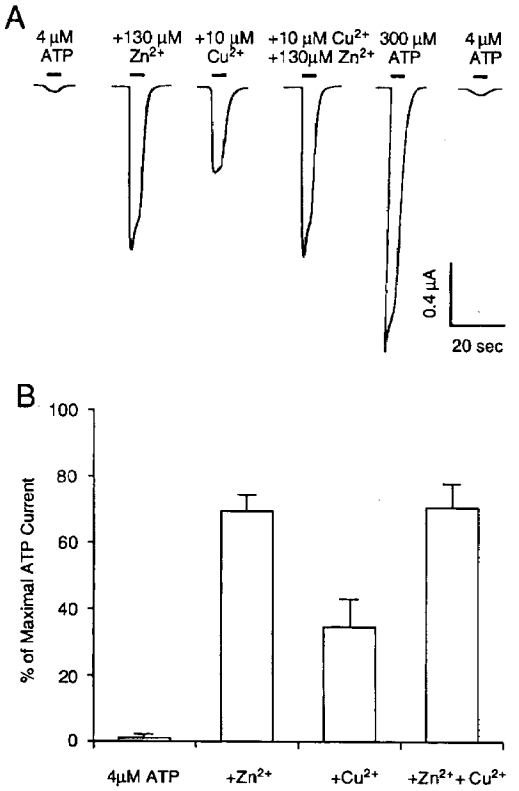

FIG. 4. Interaction of Zn^{2+} and Cu^{2+} on $P2X_2$ receptors. *A*: records illustrating effect of 130 μM Zn^{2+} and 10 μM Cu^{2+} separately and in combination on ion current activated by 4 μM ATP. Note that 300 μM ATP-activated current was much greater in amplitude than that produced by 4 μM ATP in the presence of either 130 μM Zn^{2+} or Cu^{2+} and Zn^{2+} applied together. *B*: bar graph illustrating the average potentiation of 4 μM ATP-activated current (normalized to that activated by 300 μM ATP) by 130 μM Zn^{2+} and 10 μM Cu^{2+} separately and in combination. The average potentiation of ATP-activated current produced by Cu^{2+} and Zn^{2+} applied together was not different from that produced by Zn^{2+} applied alone (Student's *t*-test, $P > 0.25$, $n = 5$).

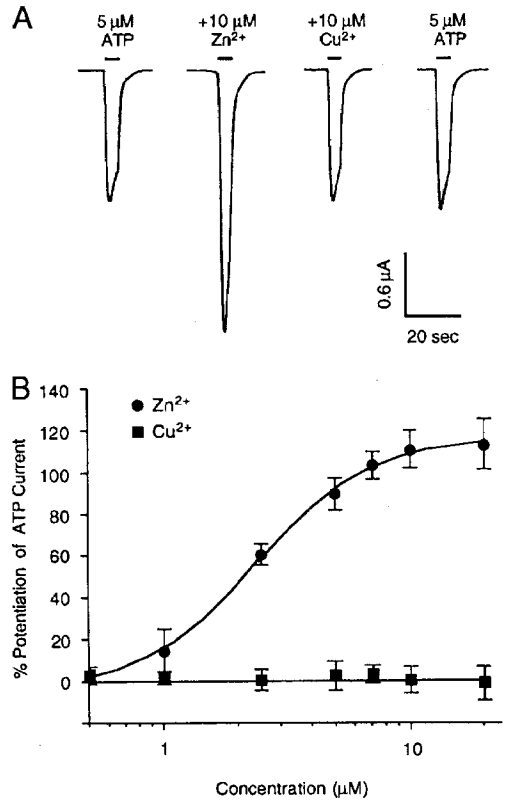

FIG. 5. Potentiation by Zn^{2+} but not by Cu^{2+} of ATP-activated current mediated by $P2X_4$ receptors. *A*: records of current activated by 5 μM ATP in the absence and the presence of 10 μM Zn^{2+} or Cu^{2+}. Records are sequential current traces (from left to right) obtained from a single oocyte. *B*: concentration-response curves for Zn^{2+} (●) and Cu^{2+} (■) potentiation of current activated by 5 μM ATP. Each point is the average of 5–9 cells; error bars not visible are smaller than the size of the symbols. The sigmoid curve shown for Zn^{2+} is the best fit of the data to the equation described in METHODS. Fitting the data to this equation yielded an EC_{50} of 2.4 ± 0.2 μM, a slope factor of 1.8, and an E_{max} of 214 ± 12% of control. The data for Cu^{2+} could not be fitted to the equation in METHODS; the line shown for these data is a least-squares plot.

5 μM ATP (4.4 μM), which produces marked potentiation of ATP-activated current. As shown in Fig. 7*B*, there was no difference in the percent potentiation by 5 μM Zn^{2+} of 5 μM ATP-activated current at membrane holding potentials between −80 and +20 mV (ANOVA, $P > 0.1$, $n = 4$). Furthermore, Zn^{2+} did

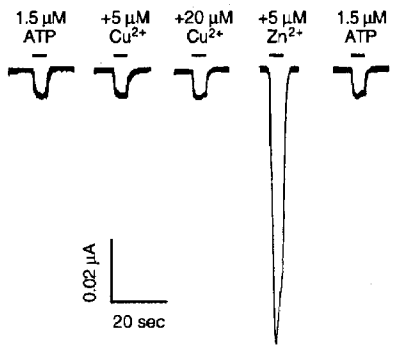

FIG. 6. Effect of Cu^{2+} and Zn^{2+} on the current activated by a near-threshold concentration of ATP in $P2X_4$ receptors. Records of current activated by 1.5 μM ATP in the absence and the presence of 5 and 20 μM Cu^{2+} and 5 μM Zn^{2+}, respectively. Records are sequential current traces (from left to right) obtained from a single oocyte.

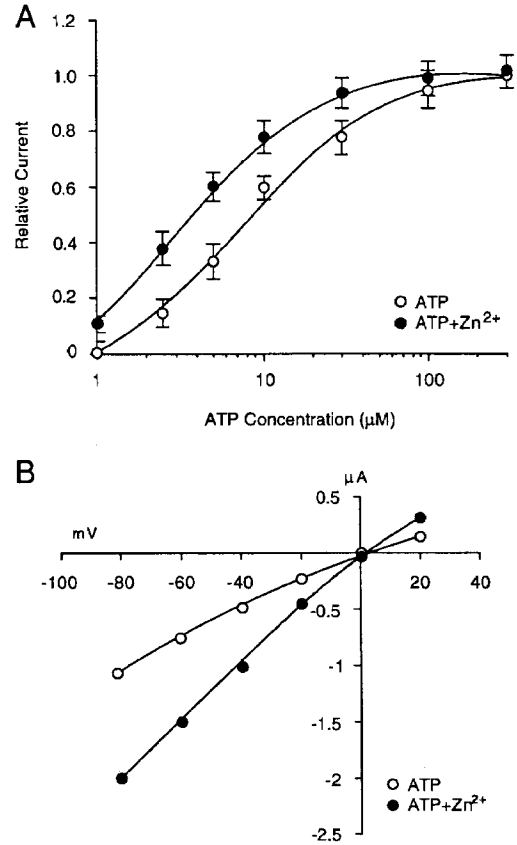

FIG. 7. Investigation of the mechanism of Zn^{2+} potentiation of ATP-activated current mediated by $P2X_4$ receptors. *A*: concentration-response for ATP-activated current in the absence (○) and the presence (●) of 5 μM Zn^{2+}. Amplitude is normalized to the current activated by 300 μM ATP in the absence of Zn^{2+}. Each point is the average of 5–8 cells. The curves shown are the best fit of the data to the equation given in METHODS. Zn^{2+} significantly decreased the EC_{50} value for ATP from 6.7 ± 1.3 μM in the absence of Zn^{2+} to 2.8 ± 0.2 μM in the presence of 5 μM Zn^{2+} (ANOVA, $P < 0.01$). *B*: current-voltage relationship for currents activated by 5 μM ATP in the absence (○) and the presence (●) of 5 μM Zn^{2+} at membrane potentials between −80 and +20 mV in a single oocyte. The average reversal potential of current activated by 5 μM ATP was −0 ± 4 mV in the absence and 1 ± 5 mV in the presence of 5 μM Zn^{2+}; these values are not significantly different (Student's *t*-test, $P > 0.1$, $n = 4$). In addition, the percentage potentiation of ATP-activated current by Zn^{2+} was not significantly different at holding potentials from −80 to +20 mV (ANOVA, $P > 0.1$, $n = 4$). Membrane potential was held at each value for 1 min before application of ATP.

not change the reversal potential of ATP-activated current (Student's *t*-test, $P > 0.1$, $n = 4$).

To evaluate a possible interaction of Cu^{2+} with the Zn^{2+} site on the $P2X_4$ subunit, we examined whether Cu^{2+} could affect Zn^{2+} potentiation of ATP-activated current. As shown in Fig. 8*A*, Cu^{2+} did not alter either ATP-activated current or Zn^{2+} potentiation of ATP-activated current. The average potentiation of ATP-activated current produced by 10 μM Zn^{2+} was 211 ± 8% of control in the absence of Cu^{2+} and 212 ± 9% of control in the presence of 10 μM Cu^{2+}; these values are not significantly different (Student's *t*-test, $P > 0.5$, $n = 5$; Fig. 8*B*).

DISCUSSION

Both Cu^{2+} and Zn^{2+} may be involved in the modulation of CNS function, as both ions were demonstrated to be widely

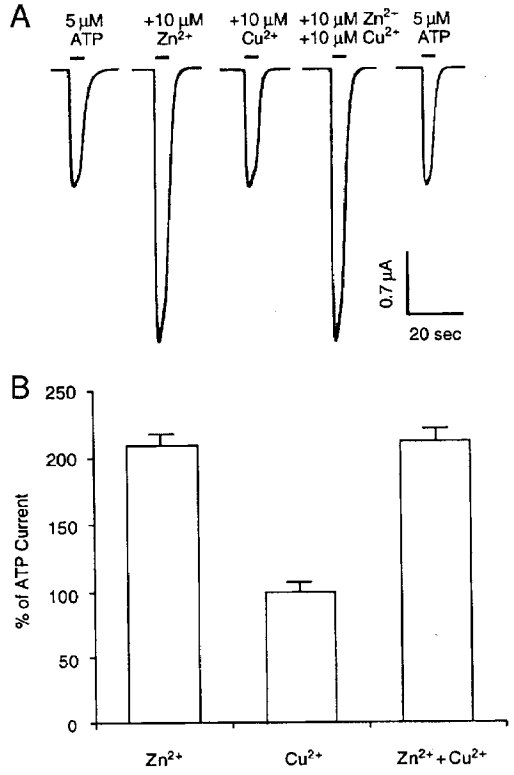

FIG. 8. Effect of Cu^{2+} on Zn^{2+} potentiation of ATP-activated current in $P2X_4$ receptors. *A*: records illustrating similar enhancement by 10 μM Zn^{2+} of currents activated by 5 μM ATP in the absence and the presence of 10 μM Cu^{2+}. Records are sequential current traces (from left to right) obtained from a single oocyte. *B*: bar graph illustrating the average potentiation of 5 μM ATP-activated current by 10 μM Zn^{2+} in the absence and the presence of 10 μM Cu^{2+}. Cu^{2+} did not alter the average potentiation of ATP-activated current produced by Zn^{2+} (Student's *t*-test, $P > 0.5$; $n = 5$).

distributed in brain (Barden 1971; Frederickson 1989; Kozma et al. 1981; Szerdahelyi and Kása 1986) and can be released on stimulation (Assaf and Chung 1984; Kardos et al. 1989). Previous studies revealed that these ions could produce similar modulation of the function of P2X receptors. For instance, micromolar concentrations of Cu^{2+} and Zn^{2+} potentiate ATP-activated current in rat nodose ganglion neurons (Li et al. 1993, 1996a). By contrast, micromolar concentrations of Cu^{2+} and Zn^{2+} inhibit ATP-activated current mediated by $P2X_7$ receptors (Virginio et al. 1997). However, this study provides evidence that Cu^{2+} and Zn^{2+} differentially regulate the function of $P2X_2$ and $P2X_4$ receptors.

Low micromolar concentrations of Zn^{2+} were previously reported to potentiate ATP-activated current mediated by $P2X_2$ receptors by increasing the apparent agonist affinity (Brake et al. 1994; Wildman et al. 1998). In the current study on $P2X_2$ receptors, Zn^{2+} potentiated the current activated by 50 μM ATP with an EC_{50} value of 19.6 μM. Similarly, Cu^{2+} markedly potentiated current activated by 50 μM ATP in $P2X_2$ receptors, with an EC_{50} value of 16.3 μM, which did not differ significantly from the EC_{50} value for Zn^{2+}. Cu^{2+} shifted the ATP concentration-response curve to the left in a parallel manner, decreasing the EC_{50} value for ATP, as was found for Zn^{2+}. These results suggest that Cu^{2+} and Zn^{2+} may facilitate the function of $P2X_2$ receptors via a common mechanism, perhaps through a common binding site. If this is the case, when this site is saturated by Zn^{2+}, Cu^{2+} should not be able to further enhance the function of the receptor, as was found in this study. The amplitude of ATP-activated current in the presence of a maximally effective concentration of Zn^{2+} was not increased further by addition of Cu^{2+}. This was not due to a "ceiling effect," that is, the ATP-gated receptors were not already maximally activated in the presence of Zn^{2+} because 300 μM ATP activated a current of substantially greater amplitude. Thus Cu^{2+} and Zn^{2+} most probably act on a common site on the ATP-gated, receptor-channel complex. The location of the Cu^{2+}–Zn^{2+} site cannot be precisely identified at present, but results of current-voltage experiments suggest that this site is beyond the influence of the membrane electrical field (Woodhull 1973) because the effects of both ions were not voltage dependent between −80 and +20 mV.

To date, $P2X_4$ receptors were cloned from rat brain (Bo et al. 1995; Séguéla et al. 1996; Soto et al. 1996), rat superior cervical ganglion (Buell et al. 1996b), and human brain (Garcia-Guzman et al. 1997), and Zn^{2+} potentiation of ATP-activated current was reported for $P2X_4$ receptors isolated from rat brain (Séguéla et al. 1996; Soto et al. 1996) and human brain (Garcia-Guzman et al. 1997). In this study, in oocytes expressing $P2X_4$ receptors cloned from rat superior cervical ganglion (Buell et al. 1996b), low micromolar concentrations of Zn^{2+} potentiated ATP-activated current. Like $P2X_2$ receptors, Zn^{2+} enhanced ATP receptor function by producing a parallel leftward shift in the ATP concentration-response curve. These results are consistent with previous studies in which Zn^{2+} was shown to induce a leftward shift of the concentration-response curve for ATP in $P2X_4$ receptors isolated from human brain (Garcia-Guzman et al. 1997). In addition, the effect of Zn^{2+} on $P2X_4$ receptors was not voltage dependent, suggesting that its site of action is not influenced by the membrane electrical field. In contrast to the effect of Zn^{2+} on $P2X_4$ receptors, 0.5–50 μM Cu^{2+} did not significantly affect ATP-activated current in oocytes expressing $P2X_4$ receptors. Furthermore, Cu^{2+} did not alter Zn^{2+} potentiation of ATP-activated current, suggesting that it does not interact with the Zn^{2+} site over this concentration range.

Cu^{2+} and Zn^{2+} at low micromolar concentrations were previously reported to differentially modulate glycine receptors in rat olfactory bulb neurons (Trombley and Shepherd 1996). The effects of Cu^{2+} and Zn^{2+} on glycine receptors are dependent on the state of the receptor; both Cu^{2+} and Zn^{2+} had no effect on the desensitized component of the current evoked by high concentrations of glycine, but Zn^{2+} dramatically potentiated and Cu^{2+} inhibited the current activated by nondesensitizing concentrations of glycine. Similarly, the results of this study suggest important differences in the modulatory sites of Cu^{2+} and Zn^{2+} on the $P2X_2$ and $P2X_4$ subunits. The observation that Cu^{2+} and Zn^{2+} interact with the site on the $P2X_2$ receptor with similar affinity may indicate that the dimensions of this site, or the dimensions of the path of access to the site, are sufficient to accommodate both ions. The lack of effect of Cu^{2+} on the $P2X_4$ subunit may thus indicate that the dimensions or path of access to the site are not sufficiently large to accommodate the larger Cu^{2+} ion. An alternative possibility is that on the $P2X_2$ subunit there are separate sites for Cu^{2+} and Zn^{2+} but that both sites affect receptor function via a common mechanism (e.g., binding of either ion to its site produces the same conformational change in the receptor, increasing its affinity for ATP). If this is the case, then the inability of Cu^{2+}

to enhance ATP-activated current mediated by $P2X_4$ receptors may be due to the absence of the Cu^{2+} site on this subunit. Future studies may be able to distinguish between these two alternatives.

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Present address and address for reprint requests: C. Li, Dept. of Cell Biology, Astra Arcus USA, Inc., Three Biotech, One Innovation Drive, Worcester, MA 01605.

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